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Clinical Significance of Serum p53 Antibody Detection on Chemosensitivity Assay in Human Colorectal Cancer

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Background and Objectives: Alteration of the *p53* gene product occurs frequently during progression of colorectal cancer. Recently, mutated p53 protein was found to induce the production of anti-p53 antibodies in the serum of patients. The purpose of this study was to evaluate the relationship between p53 status in serum and chemosensitivity in resectable colorectal cancer patients.

Methods: A total of 22 patients with primary colorectal cancer who underwent surgical treatment were examined for chemosensitivity with iable tumor samples using the Histoculture Drug Response Assay (HDRA). Serum samples of these patients for p53 antibodies were obtained before tumor resection and assayed in duplicate using an enzyme-linked immunosorbent assay kit.

Results: The inhibition index of 5-fluorouracil and *cis*-diamminedichloroplatinum (CDDP), determined by the HDRA method, in the seropositive group was significantly lower than that in the seronegative group ($P < 0.01$). Furthermore, significant statistical differences in chemosensitivity to 5-fluorouracil and CDDP were revealed depending on the presence of serum p53 antibodies.

Conclusions: Detection of serum p53 antibodies, which reflects p53 mutations in tumor tissue, is a simpler method which correlates with chemosensitivity and may contribute to the selection of favorable chemotherapeutic strategies for colorectal cancer.

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KEY WORDS: **chemosensitivity; serum p53 antibodies; colorectal cancer**

INTRODUCTION

p53 frequently undergoes mutation in a variety of human cancers [1] and accumulates in cancer cells with gene abnormalities. To date, p53 mutations have been detected by molecular analysis and p53 protein accumulation by immunohistochemical examination of tumor specimens. However, since these methods are not suitable for daily analysis, a simpler method for p53 mutation detection is necessary. Recently, p53 protein overexpression was found to induce antibody production in patient serum and the detection of serum antibodies to p53 protein has been made easier by the enzyme-linked immunosorbent assay (ELISA) [2–4].

p53 protein has been shown to play an important role in the response to DNA damage induced by chemotherapeutic agents, such as *cis*-diammindichloroplatinum (CDDP) or 5-fluorouracil [5]. Moreover, several studies have reported this as a mechanism for a rapid increase in p53 protein level and the mediation of several cellular responses, including G_1 arrest via transcriptional induc-

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tion of $p21^{WAF1}$ [6], DNA damage repair via transcriptional induction of GADD45 [7], and induction of apoptosis [8]. Since mutant p53 protein has a prolonged half-life compared with the wild-type protein, these findings raise the possibility of an association between mutant p53 expression and the sensitivity or resistance of cancer cells to anticancer drugs. In the present study, we investigated the possible relationship between the presence of serum p53 antibodies induced by p53 mutation and the chemosensitivity determined by Histoculture Drug Response Assay (HDRA) [9] in patients with colorectal cancer.

MATERIALS AND METHODS

Patients and Clinical Samples

Twenty-two patients with colorectal adenocarcinoma, hospitalized at Chiba University Hospital after August 1997, were included in this study. Patients consisted of 14 males and 8 females with a mean age of 60.4 years. All patients were primary cases and underwent surgical treatment. Staging of colorectal cancer was classified according to the TNM classification [10]. Serum samples of these patients for p53 antibodies were obtained before tumor resection and stored at −80°C until assay. Tumor samples for p53 immunostaining were fixed in 10% formalin and paraffin-embedded. Viable tumor samples for chemosensitivity assay were removed from tumor specimens under sterile conditions as soon as possible after surgery and reserved at 4°C in Hanks balanced salt solution (HBSS; GIBCO, Gaithersburg, MD) for approximately 24 hr.

Serum p53 Antibody Assay

Serum p53 antibodies were assayed in duplicate using an ELISA kit (Pharmacell, Paris, France) according to the manufacturer‘s instructions. In brief, samples were diluted 100-fold, added to the wells of a microtiter plate coated with human p53 or control protein, and incubated for 1 hr. The plate was washed 4 times, and 100 μl of the conjugated second antibody were added and incubated for another hour, after which the plate was washed again and substrate solution added. After addition of the stop solution, development of color was read at 450 nm by a microtiter plate reader. Results showing both an index of 1.1 or more and a net absorbance ratio of 1.6 or more were considered positive.

p53 Protein Immunostaining

The presence of p53 protein in tumor was detected by anti-p53 antibody DO-7 and compared with detection of p53 antibodies in serum of colorectal cancer patients. In brief, 4 μm sections on sialian-coated slides were made from formalin-fixed, paraffin-embedded tissue. The paraffin was removed in xylene, and the slides were dehydrated through graded concentrations of alcohol. After deparaffinization, endogenous peroxidase blocking (0.1% hydrogen peroxidase in 95% methanol for 5 min), sections were placed in 0.01 M citric acid and processed in a microwave oven at 600 W for 25 min. Abolishing nonspecific binding (10% carrier protein solution for 20 min) and incubation with primary monoclonal antibody (DO-7, diluted 1:50; Novocastra, Newcastle, UK) were performed at 4°C for 12 hr. After washing in PBS, slides were incubated with the secondary antibody (biotinylated mouse anti-mouse antibody, diluted 1:500; Dako, Carpinteria, CA) at room temperature for 30 min, washed, and incubated with diaminobenzidine (DAB) solution for 5 min. After a final washing in tap water, slides were counterstained with hematoxylin for 2 min, dehydrated, cleared, and mounted. Staining was considered positive if more than 10% of the cells were clearly stained in the nucleus.

Chemosensitivity Assay (HDRA)

The HDRA was performed according to a method previously reported [9]. The chemotherapeutic drugs used included 5-fluorouracil, mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan) and CDDP (Bristol-Myers Squibb, Tokyo, Japan). The anti-cancer drugs were dissolved in RPMI-1640 medium containing 20% fetal calf serum and penicillin-streptomycin-amphotericin B; 1 ml per well of the solution was poured into a 24-well plate. The cut-off concentration of 300 μg/ml for 5-fluorouracil was chosen based on a previous report [10], with equivalent cut-off levels used for CDDP (20 μg/ml) and MMC (2 μg/ml). The collagen gel (Gel Form; Pharmacia-Upjohn, UK) was cut into 1 cm^3 pieces and placed in plate wells. Surgical specimens were cut into 10 to 15 mg pieces, weighed, and placed on the collagen gels. Six replicates were executed for the control and 4 for the treatment group. After incubation for 7 days, 100 μl of 0.06% collagenase (Sigma, St. Louis, MO) solution in HBSS and 100 μl of 0.2% MTT (Sigma) phosphate-buffered saline solution containing 50 mM sodium succinate (Wako, Osaka, Japan) were added to each well. After the plate was incubated for a further 16 hr, medium was removed and 0.5 ml per well of dimethyl sulfoxide was added to extract MTT-formazan. Two hours later, solution extracted from each well was moved to a 96-well plate and absorbance was measured by a microplate reader at 540 nm. Cases of contamination and absorbance of less than 15 per 1 g control tumor were regarded as unsuitable.

Inhibition index was calculated as follows: Inhibition index (%) = $(1 - A/B) \times 100$, where A is the mean absorbance of treated wells per 1 g tumor and B is the mean absorbance of control wells per 1 g tumor. A drug with 50% or higher inhibition index was regarded as in vitro chemosensitive.

TABLE I. Association Between the Presence of Serum p53 Antibodies and Clinicopathological Features

	Serum p53 antibodies		
	Positive	Negative	*P* value
Number	13	9	
Age (years)	63.4	56.1	0.11
Gender (male:female)	7:6	7:2	0.49
TNM stage			
II	6	2	
III	4	2	0.28
IV	3	5	
p53 immunostaining			
+	12	0	<0.001
−	1	9	

Statistical Analysis

Chi-square analysis was applied in order to determine the significance between the presence of p53 antibodies and clinicopathological features. Student's *t*-test or the chi-square test was used to determine the significance between the two groups. *P* values less than 0.05 were considered significant.

RESULTS

Association Between the Presence of Serum p53 Antibodies and Clinicopathological Features

According to the presence of serum p53 antibodies, all patients were divided into seropositive and seronegative groups (Table I). No significant differences between seropositive and seronegative groups were observed with age ($P = 0.11$), gender ($P = 0.49$), or clinical staging by TNM classification ($P = 0.28$) [11]. However, 12 (92.3%) out of 13 seropositive patients showed p53 positive staining, and all seronegative patients revealed negative staining of tumor tissue, thus demonstrating a significant correlation between immunostaining for p53 protein and the presence of serum p53 antibodies($P < 0.001$).

Results of Inhibition Index According to Serum p53 Antibody Status

Figure 1 shows the overall inhibition index determined by the HDRA for three chemotherapeutic agents, 5-fluorouracil, CDDP, and MMC, according to the presence of serum p53 antibodies. Inhibition rates of 5-fluorouracil, CDDP, and MMC in seropositive patients were 23.0 ± 19.0%, 17.1 ± 13.8%, and 42.2 ± 26.8%, respectively, and in seronegative patients 62.3 ± 7.0%, 54.1 ± 18.3%, and 63.5 ± 10.1%, respectively. Inhibition rates of 5-fluorouracil and CDDP ($P < 0.01$) were significantly lower in the seropositive group compared to the seronegative group.

Chemosensitivity Depending on the Presence of Serum p53 Antibodies

Table II presents the relationship between chemosensitivity in colorectal cancer patients to 5-fluorouracil, CDDP, and MMC and the presence of serum p53 antibodies and lists the numbers of chemosensitive (+) or insensitive (−) patients.

Chemosensitivity was regarded as positive when a drug had 50% or higher inhibition index as tested by the previously described methods. Statistically significant differences ($P < 0.001$) in drug chemosensitivity to 5-fluorouracil and CDDP were revealed depending on the presence of serum p53 antibodies.

DISCUSSION

The optimal treatment for advanced colorectal cancer has yet to be established. This disease has been regarded as poorly chemosensitive, and the only active agent of choice has been 5-fluorouracil, whose reported objective response rate has been only 10% to 15%, with a median overall survival of 5 years [12]. Although improvement of alternating regimens for colorectal cancer is important, it is of prime importance to select nonresponder cases for standard chemotherapy in order to exclude noneffective treatment regimens which aggravate the quality of life. Individualization of colorectal cancer chemotherapy by a clinically useful drug response assay is significant; however, the development of a simpler method reflecting the results of chemosensitivity assay is required. It has been reported that wild-type p53 protein correlates with apoptosis induction if followed by anticancer drugs [13], whereas mutated type p53 tends to suppress apoptosis of cancer cells [14]. Wild-type p53 has a short half-life and is undetectable in tissues; in contrast, mutated p53 has a greatly extended protein half-life, permitting immunohistochemical detection; thus, tumors exhibiting p53 immunostaining certainly have mutated p53 protein [15]. Accumulation of p53 protein to a level detectable by immunohistochemistry, i.e., the overexpression of mutant p53 protein, has been found to induce a specific response in cancer patients [16]. Immunologic p53 protein results in the production of anti-p53 antibodies in patient serum. Consequently, p53 protein detected in serum by the previously described ELISA method is regarded as the mutated type. Moreover, p53-seropositive patients appear to have mutated p53 protein in serum with a fair degree of certainty. In our study of 22 colorectal cancer patients, a significant correlation was evidenced between results of immunohistochemical analysis and the presence of serum p53 antibodies, which indicates that the presence of serum p53 antibodies reflects p53 mutation in tumor tissues.

Overexpression of mutated p53 leads to a dramatic

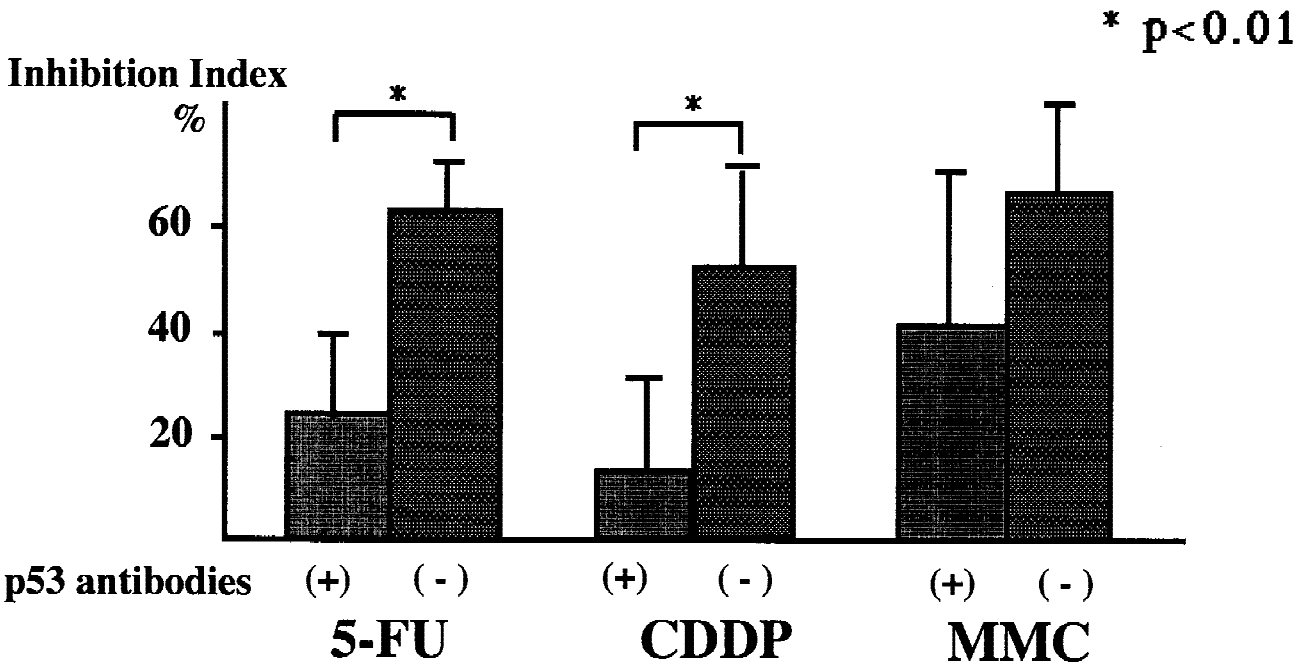

Fig. 1. Results of inhibition index according to serum p53 antibody status.

TABLE II. Chemosensitivity Depending on the Presence of Serum p53 Antibodies

Serum p53 antibodies	5-Fluorouracil (+/−)[c]	CDDP[a] (+/−)[c]	MMC[b] (+/−)[c]
+	0/13	0/13	6/7
−	9/0	7/2	6/3
P value	<0.001	<0.001	0.61

[a]CDDP, *cis*-diamminedichloroplatinum.
[b]MMC, mitomycin C.
[c]Numbers of patients chemosensitive (+) or insensitive (−).

increase in cellular resistance to treatment with chemotherapeutic compounds, implying that tumor cells acquire drug resistance through mutations that interfere with apoptosis [13]. Several recent papers have reported on the relationship between expression of mutated p53 and chemosensitivity assay or clinical prognosis. Most mutant p53-positive tumors of gastric cancer were reported to show low chemosensitivity rates [17]. Bergh et al. [18] reported that p53 status relates to prognosis and the effect of adjuvant therapy by complete sequencing of the *p53* gene in primary breast cancer. Moreover, lymph node-positive patients with p53 mutations were reported to have significantly shorter survival compared to those without p53 mutations [18]. It has also been reported that high expression of the p53 oncoprotein is a favorable prognostic factor in non-small-cell lung cancer patients [19] and that p53 expression is associated with poor prognosis in bladder cancer [20].

Although the mechanism by which *p53* gene mutation affects clinical outcome is unknown, two pathways, dependent on *p53* mutation or independent, for induction of apoptosis in cancer chemotherapy have been suggested [13]. Since most chemotherapeutic agents induce apoptosis dependent on *p53* mutations [21], p53 status has been suggested to be a predictor of chemosensitivity. In our study, it was found that p53 mutation of tumor cells could be easily speculated by serum investigation and that p53-seropositive patients induce poor chemosensitivity. These findings may be a contributing factor to preoperative serum p53 antibody detection for colorectal cancer patients. While it is necessary to explore the biologic activities of different classes of p53 mutation, detection of serum p53 antibodies in colorectal cancer may be a useful parameter in the selection of postoperative adjuvant chemotherapy. The relationship between the presence of serum p53 antibodies and their prognostic effects and the clinical significance of serum p53 antibody detection remain to be investigated.

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